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# Identification and Partial Purification of a Factor That Stimulates Calcium-Dependent Proteases†

George N. DeMartino* and Donald K. Blumenthal

ABSTRACT: We have identified and partially purified from bovine brain a factor which stimulates the activities of two rat liver calcium-dependent proteases. The factor, which is physically and functionally distinct from calmodulin, is heat stable and has a low apparent molecular weight ($M_r$ 20000). The factor stimulates rates of proteolysis up to 25-fold but does not alter the calcium sensitivity of either protease. We have called this factor calcium-dependent protease regulator.

Recently we have been studying two rat liver cytoplasmic proteases which are completely dependent on calcium for activity. These enzymes are distinguishable by different calcium concentration requirements (DeMartino, 1981). Because the activities of many calcium-dependent enzymes are regulated by the calcium-binding protein calmodulin (Klee et al., 1980; Cheung, 1980), we examined the effects of calmodulin on the calcium-dependent proteases. In our initial experiments (DeMartino & Kuers, 1981) both proteases were greatly stimulated by samples of bovine brain calmodulin which had been prepared in a manner similar to previously published methods (Dedman et al., 1977). However, in subsequent experiments, neither protease was affected by highly purified bovine brain calmodulin which had been prepared by affinity chromatography on fluphenazine–Sepharose. Despite the different protease stimulatory activities of the two calmodulin preparations, each was able to activate the calmodulin-dependent enzyme, myosin light chain kinase. These results suggested that the factor responsible for the protease stimulatory activity in the original preparation was distinct from calmodulin. The purpose of the present work was to determine whether this possibility was correct and, if so, to identify and to characterize this stimulatory activity.

## Materials and Methods

*Preparation of Rat Liver Calcium-Dependent Proteases.* Calcium-dependent proteases from rat liver were prepared as described previously (DeMartino, 1981). After isolation by DE52 ion-exchange column chromatography, each protease was dialyzed against 50 mM Tris-HCl,[1] pH 7.5, 0.5 mM

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; Mops, 3-(*N*-morpholino)propanesulfonic acid; CDPR, calcium-dependent protease regulator; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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EGTA, and 0.5 mM DTT and stored at −70 °C. Prior to assay, aliquots of enzyme were thawed and dialyzed for 18 h against 6000 volumes of 5 mM Tris-HCl, pH 7.5, and 0.5 mM DTT. As reported previously (DeMartino, 1981), calcium-dependent protease peak I required approximately 5–10 $\mu$M $Ca^{2+}$ for half-maximal activity, and calcium-dependent protease peak II required approximately 150 $\mu$M $Ca^{2+}$ for half-maximal activity.

*Preparation of Heat-Stable Extracts from Bovine Brain.* Frozen bovine brains were obtained from Pel Freez. Brains were allowed to partially thaw at 4 °C for 12 h prior to homogenization. Except where noted, all remaining steps were carried out at 0–4 °C. Two brains (~700 g) were minced and homogenized in 700 mL of 10 mM Tris-HCl, pH 7.5, 0.5 mM DTT, and 0.5 mM EGTA (buffer A) with a Waring blender (90 s, high speed). The homogenate was centrifuged for 30 min at 14000*g*. The supernatant fraction was retained, and pellets were rehomogenized with an additional 700 mL of buffer A. After recentrifugation, the supernatant fractions from both centrifugations were combined and added to 300 mL of water heated to 90 °C. The temperature of the extract was raised to 90 °C and maintained for 10 min with constant stirring. After the extract was cooled to 4 °C in an ice bath, the heat-precipitated proteins were removed by centrifugation for 30 min at 14000*g*. The clear, yellow supernatant contained approximately 500 mg of protein. This amount of protein represented less than 5% of the total protein present in the supernatant of the original homogenates.

*Ion-Exchange Column Chromatography of Heat-Stable Brain Extracts.* The heat-stable supernatant fraction was adjusted to pH 7.5 and added to 50 g of diethylaminoethylcellulose (DE52, Whatman) equilibrated with buffer A. After being gently mixed for 60 min, the slurry was poured into a 25 × 5 cm column and washed thoroughly with buffer A until no protein was detected in the column effluent. Approximately 60% of the protein in the heat-stable supernatant did not bind to the DE52. The bound protein was eluted from the column with a linear gradient (600 mL, 0.0–0.6 M) of KCl in buffer A. Aliquots of each of the 10-mL fractions were assayed for protein and for the ability to stimulate protease and myosin light chain kinase activities as described in the text.

*Sephadex G-75 Column Chromatography.* Ascending gel filtration chromatography was carried out with Sephadex G-75 in a 95 × 2.5 cm column. The gel was equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, and 0.5 mM EGTA. Five-milliliter fractions were collected. The column was calibrated with proteins of known molecular weights (ovalbumin, $M_r$ 43 000; chymotrypsinogen A, $M_r$ 25 000; ribonuclease A, $M_r$ 13 700), and the void volume was determined with blue dextran.

*Fluphenazine–Sepharose Affinity Chromatography.* Fluphenazine (a generous gift of S. J. Lucania, Squibb Institute for Medical Research) was coupled to Sepharose by using epoxy-activated Sepharose 4B (Sigma) by a procedure similar to that previously described (Kakiuchi et al., 1981). The affinity resin was equilibrated with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl and 2 mM $CaCl_2$. After sample application, the column was washed with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 0.2 mM $CaCl_2$. Bound material was eluted with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM EGTA. The column dimensions were 1 × 2 cm, and fractions were 2.5 mL.

*Measurement of Protease Activity.* Protease activity was measured by the hydrolysis of radioactively labeled protein substrates to acid-soluble peptides as described previously (DeMartino, 1981). Substrates included [*methyl*-$^{14}$C]globin, [*methyl*-$^{14}$C]casein, and [*methionyl*-$^{35}$S]-$\beta$-actin (a generous gift of Dr. D. E. Croall). Each assay was buffered with Tris-HCl at the pH and ionic strength given in the legends to the figures and contained calcium-dependent protease, 1.5 mM DTT, $CaCl_2$ of appropriate concentration, and substrate [either 3 $\mu$g of [*methyl*-$^{14}$C]globin (17 000 cpm), 10 $\mu$g of [*methyl*-$^{14}$C]casein (20 000 cpm), or 5 $\mu$g of [*methionyl*-$^{35}$S]-$\beta$-actin (12 000 cpm)] in a final volume of 140 $\mu$L. Specific conditions and other additions are detailed in the legends. With each assay condition a series of control reactions were also performed. These included (1) assays with no enzyme protein, (2) assays with no calcium, and (3) assays with excess EGTA. No protease activity was ever detected with any of these controls. Assays were carried out at 25 °C. The amount of enzyme protein and the time of incubation were adjusted such that all reaction rates were linear with respect to time. At the end of the incubation, reactions were terminated by addition of 700 $\mu$L of 10% trichloroacetic acid containing 2 mg/mL bovine serum albumin. After 3 h at 4 °C, the samples were centrifuged, and 400 $\mu$L of the supernatant was counted by liquid scintillation counting. Protease activity is expressed as the counts per minute in such a sample after subtraction of counts per minute in the appropriate control samples detailed above. In a given experiment, each assay was performed in triplicate, and there was normally less than 5% variation among replicates.

*Myosin Light Chain Kinase Assay.* Myosin light chain kinase activity was assayed by measuring rates of $^{32}$P incorporation into myosin P-light chain as previously described (Blumenthal & Stull, 1980). Calmodulin-deficient myosin light chain kinase purified from rabbit skeletal muscle (40 pg/mL) was incubated at 30 °C with 50 mM Mops, pH 7.0, 100 $\mu$M $CaCl_2$, 10 mM magnesium acetate, 60 $\mu$M myosin P-light chain from bovine cardiac muscle, 2 mM [$\gamma$-$^{32}$P]ATP (150–200 cpm/pmol), and the indicated quantities of each protein fraction. The ability of a protein fraction to activate myosin light chain kinase was compared to that of homogeneous bovine brain calmodulin. A value of 1.0 relative calmodulin unit was assigned to a protein fraction capable of half-maximally activating the enzyme at a protein concentration identical with that observed for half-maximal activation by purified calmodulin.

*Polyacrylamide Gel Electrophoresis.* Linear gradient (5–15%) polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate ($NaDodSO_4$) as described previously (Bednarz-Prashad & Mize, 1978). Molecular weight markers (carbonic anhydrase, $M_r$ 30 000; soybean trypsin inhibitor, $M_r$ 20 100; $\alpha$-lactalbumin, $M_r$ 14 000) were run on all gels.

*Protein Determinations.* Protein was estimated by the method of Bradford (1976) with premixed reagent purchased from Bio-Rad Laboratories. Bovine serum albumin served as the standard.

## Results

*Stimulation of Calcium-Dependent Proteases by Heat-Stable Extracts from Bovine Brain.* Highly purified bovine brain calmodulin, prepared by fluphenazine–Sepharose affinity chromatography, failed to stimulate the activities of the rat liver calcium-dependent proteases (data not shown). However, calmodulin prepared similarly to published methods (Dedman et al., 1977) stimulated each protease up to 7-fold (DeMartino & Kuers, 1981). We therefore hypothesized that a factor, distinct from calmodulin, contaminated the latter preparation and was responsible for the protease stimulatory activity. In

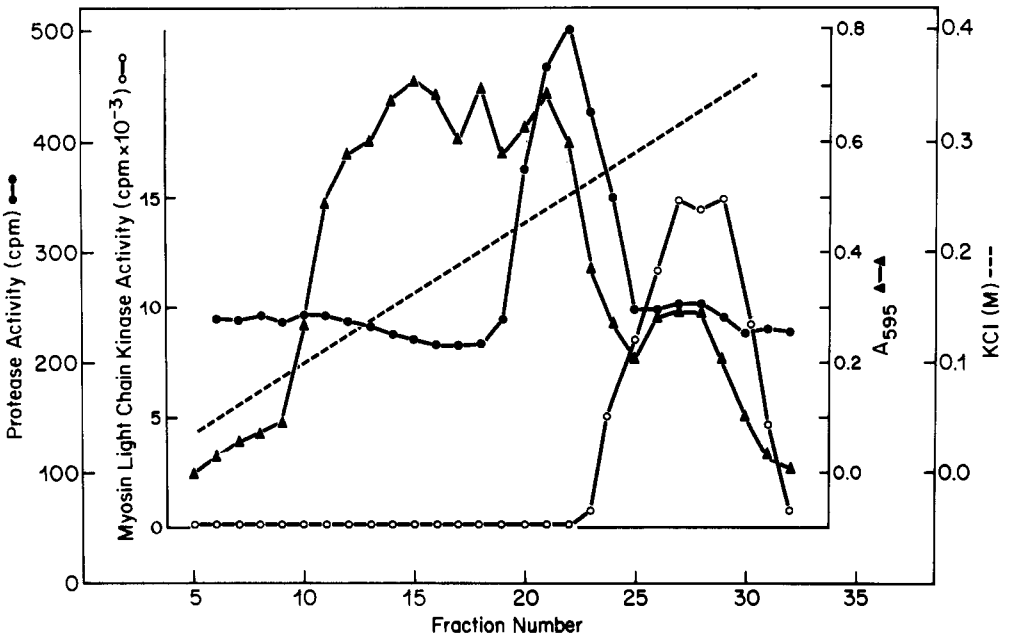


FIGURE 1: DE52 anion-exchange column chromatography of bovine brain extracts. Heat-stable extracts from bovine brain were subjected to DE52 anion-exchange chromatography as described under Materials and Methods. Aliquots (10 μL in this experiment; from 2.5 to 50 μL in other experiments; see Discussion) of the flow through and eluted fractions were added to assays of calcium-dependent protease peak II containing 20 mM Tris-HCl, pH 7.0, 1.5 mM DTT, and 890 μM $CaCl_2$ as described under Materials and Methods. Aliquots (equivalent to $1 \times 10^{-3}$ μL) of the eluted fractions were also assayed for the ability to activate calmodulin-deficient myosin light chain kinase as described under Materials and Methods. Myosin light chain kinase activity is expressed as cpm of $^{32}P$ incorporated into myosin light chains in 30 min. Assays with 3 μM known calmodulin incorporated 18 837 cpm. No stimulatory activity for either enzyme was detected in the flow-through fractions.

order to identify this putative factor, we subjected bovine brain extracts to various purification steps and monitored simultaneously the ability of these extracts to stimulate the calcium-dependent proteases and the calmodulin-dependent enzyme, myosin light chain kinase.

Calmodulin-rich, heat-stable extracts from bovine brain (see Materials and Methods) stimulated each protease approximately 2-fold. The extracts had no endogenous protease activity. Protease activity in the presence and absence of the extract was completely dependent on calcium and abolished by EGTA (data not shown).

*Separation of Protease Stimulatory Activity from Calmodulin.* The brain extracts were purified further by anion-exchange column chromatography. The eluted fractions contained a single peak of protease stimulatory activity and a single peak of myosin light chain kinase stimulatory activity. Although these activities eluted in similar regions of the KCl gradient, the peaks were not coincident (Figure 1). Under optimal assay conditions (see Discussion), protease activity was stimulated approximately 2-fold in the peak fractions.

*Fluphenazine–Sepharose Affinity Chromatography.* The results of the ion-exchange chromatography indicated that the protease stimulatory activity was indeed distinct from calmodulin. To confirm this, we chromatographed each peak of stimulatory activity from Figure 1 on a calmodulin-specific affinity column composed of fluphenazine covalently linked to Sepharose 4B. The protease stimulatory activity did not bind to the fluphenazine–Sepharose and passed directly through the column (Figure 2A; Table I). This unbound fraction did not stimulate calmodulin-deficient myosin light chain kinase (Table I). However, the myosin light chain kinase stimulatory activity from Figure 1 bound to the fluphenazine–Sepharose in the presence of calcium and was eluted with EGTA (Figure 2B; Table I). These data confirm that this protein was calmodulin. This calmodulin had properties identical with calmodulin purified by an independent technique (Blumenthal & Stull, 1982). Calmodulin had virtually no stimulatory effect on either of the calcium-dependent proteases (Table I).

Table I: Effect of Fractions from Fluphenazine–Sepharose Chromatography on Protease and Myosin Light Chain Kinase Activities[a]

| sample addition | protease act. (cpm) peak I | protease act. (cpm) peak II | myosin light chain kinase (rel units)[b] |
|---|---|---|---|
| control | 223 | 250 | <0.001 |
| X | 466 | 756 | <0.001 |
| Y | 217 | 244 | <0.05 |
| Z | 252 | 272 | 1.0 |

[a] Sample X from Figure 2A and samples Y and Z from Figure 2B were dialyzed extensively against 5 mM Tris-HCl, pH 7.5, and 0.1 mM DTT and tested for the ability to stimulate calcium-dependent proteases and myosin light chain kinase. Protease activity was assayed in 50 mM Tris-HCl, pH 7.0, 1.5 mM DTT, and 890 μM $CaCl_2$ in the presence of the indicated samples or dialysis buffer (10 μL, control). Myosin light chain kinase was assayed as described under Materials and Methods. [b] Relative units indicate the ability of the sample to activate calmodulin-deficient myosin light chain kinase relative to activation by a known concentration (3 μM) of homogeneous fluphenazine-purified calmodulin (see Materials and Methods).

*Sephadex G-75 Column Chromatography of the Protease Stimulatory Activity.* The calcium-dependent protease stimulatory activity from the DE52 column (Figure 1) was concentrated and chromatographed on Sephadex G-75. Stimulatory activity eluted as a single peak at a position corresponding to $M_r$ 20 000 and was separated from the bulk of the protein applied to the column (Figure 3). No stimulation of myosin light chain kinase was detected in the fractions containing protease stimulatory activity (data not shown).

The fractions containing protease stimulatory activity were combined, lyophilized, redissolved in a small amount of water,

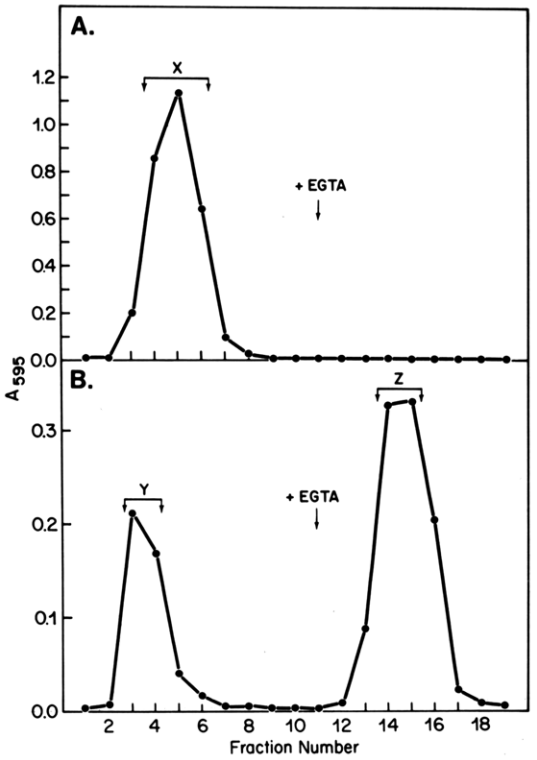


FIGURE 2: Fluphenazine–Sepharose affinity chromatography. (A) Chromatography of protease stimulatory activity. Aliquots (250 $\mu$L) of fractions 21–22 from the DE52 column (Figure 1) were combined and added to 500 $\mu$L of column buffer. This sample was applied to the column and eluted as described under Materials and Methods. Individual fractions were assayed for protein content; fractions 4–6 (X) were combined and treated as described in Table I. (B) Chromatography of myosin light chain kinase stimulatory activity. Aliquots (200 $\mu$L) of fractions 27–29 from the DE52 column (Figure 1) were combined and added to 600 $\mu$L of column buffer. This sample was applied to the column and eluted as described under Materials and Methods. Individual fractions were assayed for protein content; fractions 3–4 (Y) were combined and fractions 14–15 (Z) were combined and treated as described in Table I.

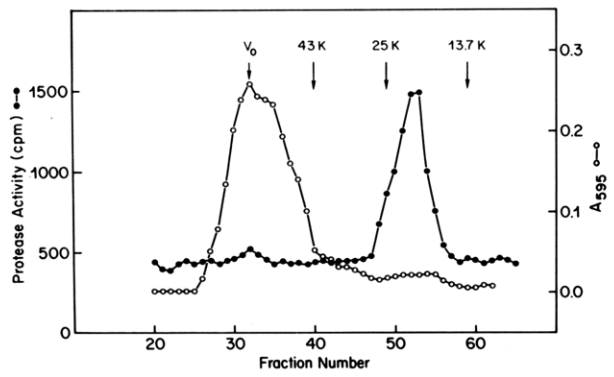


FIGURE 3: Sephadex G-75 column chromatography of protease stimulatory activity. Fractions 21–22 from Figure 1 were combined and added to ice-cold acetone. The precipitate was collected by centrifugation, dried, and redissolved in 3 mL of 50 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, and 0.5 mM DTT. This sample was chromatographed as described under Materials and Methods. Aliquots (10 $\mu$L) of the eluted fractions were tested for the ability to stimulate calcium-dependent protease peak II. Assays contained 50 mM Tris-HCl, pH 7.0, 1.5 mM DTT, 35 $\mu$M EGTA, 890 $\mu$M $CaCl_2$, and globin substrate.

and dialyzed for 48 h against two changes (6000 volumes each) of 5 mM Tris-HCl, pH 7.5, and 0.1 mM DTT. Approximately 400–500 $\mu$g of protein was normally present in this sample (from 700 g of bovine brain). Because of the small amount of protein, further purification was not attempted. The sample

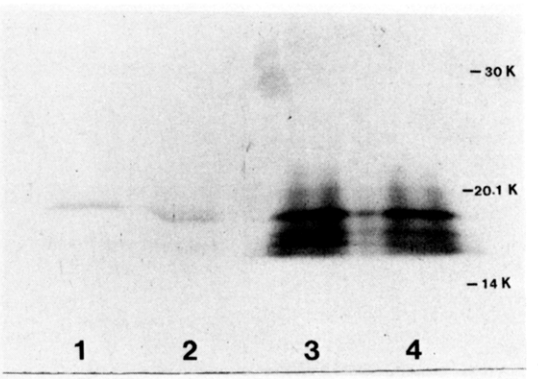


FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of CDPR and calmodulin. Samples of calmodulin and CDPR prepared as described in the text were mixed with either $CaCl_2$ or EGTA prior to addition of sample buffer and electrophoresis (8). (Lane 1) Calmodulin + EGTA; (lane 2) calmodulin + $CaCl_2$; (lane 3) CDPR + EGTA; (lane 4) CDPR + $CaCl_2$.

was designated calcium-dependent protease regulator (CDPR) and was characterized as described below.

*Characterization of CDPR.* The CDPR was electrophoresed on 5–15% linear gradient $NaDodSO_4$–polyacrylamide gels as described under Materials and Methods. Calmodulin, purified by fluphenazine–Sepharose chromatography (Figure 2B), was electrophoresed on the same gels. Each sample was mixed with either 2.5 mM EGTA or 1.25 mM $CaCl_2$ prior to electrophoresis. Calmodulin electrophoresed as a single band and migrated faster in the presence of $CaCl_2$ than in the presence of EGTA as described previously (Klee et al., 1979). The CDPR sample demonstrated at least three bands and in some preparations as many as five bands with molecular weights ranging from 22000 to 16000. The migration of these bands appeared similar in the presence of either $CaCl_2$ or EGTA (Figure 4).

CDPR stimulated the rates of protein hydrolysis by each calcium-dependent protease. These effects were observed for all substrates tested including globin, casein, and $\beta$-actin. Because the assays were linear with respect to time, both in the presence and in the absence of CDPR (data not shown), it seems unlikely that this stimulatory effect resulted indirectly from some stabilization of the proteases by CDPR. The maximum stimulation ranged from 5-fold to 25-fold depending on the particular CDPR preparation and was concentration dependent to approximately 0.5 $\mu$g of CDPR/assay (Figure 5). Proteolytic activity both in the presence and in the absence of CDPR was completely dependent on calcium. No other divalent cation tested ($Mg^{2+}$, $Mn^{2+}$, and $Cu^{2+}$ at 1 mM) could activate proteolysis (data not shown), and EGTA completely abolished all protease activity.

CDPR had no endogenous protease activity against any substrate tested (i.e., globin, casein, and $\beta$-actin). However, we also tested whether CDPR might be an exopeptidase which could act only on peptides produced by the initial action of calcium-dependent proteases. The calcium-dependent proteases were incubated with substrates for various times and then inactivated with EGTA. Subsequent addition of CDPR did not result in the production of more acid-soluble peptides. However, addition of CDPR to enzyme not treated with EGTA resulted in stimulated rates of acid-soluble peptide production (data not shown). These results suggest that CDPR is not an exopeptidase.

Stimulation of proteolytic activity by CDPR appeared to be specific for the calcium-dependent proteases. The activities of trypsin, papain, chymotrypsin, and the high molecular weight cytoplasmic protease from rat liver (DeMartino &

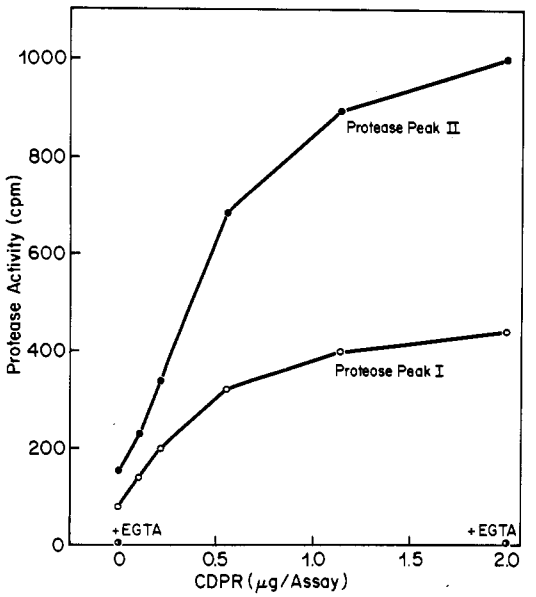


FIGURE 5: Effect of CDPR on activities of calcium-dependent protease peak I and calcium-dependent protease peak II. CDPR was prepared as described in the text. CDPR concentrations indicated were added to standard protease assays which contained either calcium-dependent protease peak I or calcium-dependent protease peak II with 30 mM Tris-HCl, pH 7.0, 1.5 mM DTT, $CaCl_2$ (peak I, 223 μM; peak II, 890 μM), and globin substrate. Data points indicated by "+EGTA" show assays identical with those described above but with the addition of 2 mM EGTA. Similar results were obtained with other CDPR preparations, but the magnitude of the maximal stimulation ranged from 5- to 25-fold. Similar results were obtained with other substrates.

Goldberg, 1979) were unaffected by CDPR (data not shown).

Despite its ability to stimulate rates of protein hydrolysis, CDPR did not alter the sensitivity of either protease to calcium (Figure 6). Thus, CDPR stimulated protease activity by the same percentage at every calcium concentration. For example, Figure 6A shows a typical experiment in which calcium-dependent protease peak II was stimulated approximately 5-fold at every calcium concentration tested. Although CDPR increased the absolute rates of proteolysis at submaximal calcium concentrations, it did not decrease the calcium concentration required for half-maximal protease activity (Figure 6B). Thus, both in the presence and in the absence of CDPR, this value was approximately 120 μM for calcium-dependent protease peak II (Figure 6B) and approximately 5–10 μM for calcium-dependent protease peak I (data not shown). Similar results were obtained at all CDPR concentrations tested and for each substrate.

Interestingly, the pH optimum for the protease activity in the presence of CDPR was significantly lower than that in its absence. This resulted in a greater stimulatory effect at pH 7.0–7.1 than at pH 7.5 or above (Figure 7). This feature was observed at all calcium concentrations and at all CDPR concentrations (data not shown).

## Discussion

Recently, we found that preparations of bovine brain calmodulin greatly stimulated the activities of two rat liver calcium-dependent proteases (DeMartino & Kuers, 1981). We now know, however, that these original calmodulin preparations, although apparently pure on the basis of gel electrophoresis, were contaminated by another factor that was totally responsible for the protease stimulation.[2] In the present

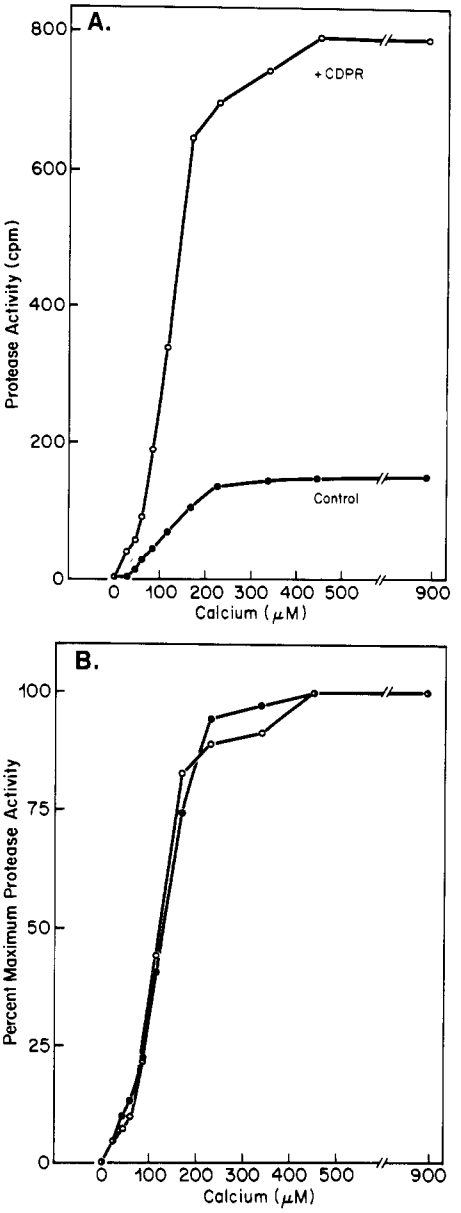


FIGURE 6: Effect of calcium concentration on CDPR-stimulated protease activity. Calcium dependent protease peak II was assayed with the various calcium concentrations indicated in the figure in the absence and presence of CDPR (0.54 μg/assay). Assays contained 30 mM Tris-HCl, pH 7.0, 1.5 mM DTT, and globin substrate. Panel A shows protease activity. In panel B, the maximum protease activity under each condition in panel A (i.e., in the presence and absence of CDPR) was set at 100%, and the other activities were expressed as a percentage of that value. Similar results were obtained with other substrates.

report, we have demonstrated the isolation of this factor, termed calcium-dependent protease regulator (CDPR). We have also demonstrated that CDPR is physically and functionally distinct from calmodulin. For example, the two activities are separated by ion-exchange chromatography. CDPR did not activate the calmodulin-dependent enzyme, myosin light chain kinase, nor did it bind to the calmodulin affinity medium, fluphenazine–Sepharose. On the other hand, calmodulin could not activate the calcium-dependent proteases. In addition, these proteases were not adsorbed to calmodulin–Sepharose,[2] a property exhibited by many calmodulin-dependent enzymes.

Although we do not know the mechanism by which CDPR stimulates protease activity, our results appear to exclude several trivial explanations for this effect. For example, we

[2] G. N. DeMartino and D. K. Blumenthal, unpublished observations.

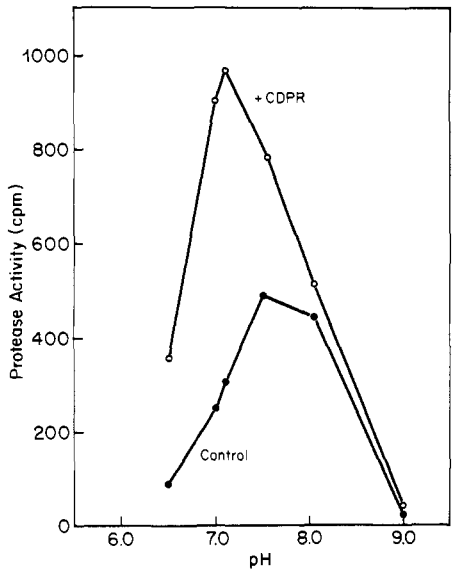


FIGURE 7: Effect of pH on CDPR-stimulated protease activity. Calcium-dependent protease peak II was assayed in the presence and absence of CDPR (0.24 μg/assay). Assays contained 50 mM Tris-HCl adjusted to the appropriate pH, 1.5 mM DTT, and 890 μM $CaCl_2$.

were unable to demonstrate any endogenous proteolytic activity in CDPR; it could not degrade casein, globin, or β-actin, nor could it further degrade partial proteolytic digests of these substrates produced by the action of calcium-dependent proteases. The apparent specificity of CDPR for the calcium-dependent proteases further argues against exopeptidase activity in CDPR. For example, if present, exopeptidase activity should have been evident after the initial action of other proteases. It also seems unlikely that CDPR stimulated proteolysis indirectly, for example, by rendering substrates more susceptible to degradation by promoting their denaturation. Such indirect stimulation should have been observed by using other proteases but was not. In addition, similar degrees of stimulation were observed with three different substrates, two of which (globin and β-actin) were already highly denatured.

Some of our data indicate that the mechanism of protease stimulation by CDPR is not similar to that by which calmodulin stimulates other enzymes. The generally accepted mechanism for calmodulin action involves two sequential and reversible binding steps: calmodulin first binds calcium, and this complex then associates with inactive enzymes to form an activated calcium–calmodulin–enzyme complex. A direct consequence of this mechanism is that calmodulin dramatically reduces the concentration of calcium required for maximum activity of an enzyme (Brostrom & Wolff, 1981). CDPR, on the other hand, stimulated rates of proteolysis but did not increase the calcium sensitivity of either protease. In addition, although the proteases were completely dependent on calcium, they exhibited significant activity in the absence of added CDPR. Thus, these data may indicate that calcium binds to the proteases directly to activate the enzymes and that CDPR further stimulates the calcium–protease complex without itself binding calcium.

We experienced considerable difficulty in determining the appropriate assay conditions to demonstrate protease stimulatory activity in the cruder extract fractions (i.e., in the heat-stable extracts and in fractions from the DE52 column). This resulted principally because relatively high extract concentrations were required in order to detect the small amounts of CDPR. In addition to CDPR the crude fractions contained many proteins which competed with globin as substrates for the proteases and could thus artifactually decrease apparent protease activity. We typically had to carry out assays over a wide range of extract concentrations; low extract concentrations often did not contain sufficient CDPR to stimulate proteolysis, but very high extract concentrations interfered with the assay. The concentration range in which we could demonstrate protease stimulatory activity was often quite narrow and varied appreciably from preparation to preparation. We were able to obtain high stimulation without significant interference only after the Sephadex G-75 chromatography step when most of the contaminating protein had been removed from the CDPR sample.

For unknown reasons, the maximum degree of stimulation varied among CDPR preparations (5–25-fold stimulation). However, the apparent concentration of CDPR required for protease stimulation was fairly consistent among preparations. Under the assumption that CDPR is a protein and that all of the protein after the Sephadex G-75 step was CDPR with a molecular weight of 20 000, half-maximal stimulation was achieved at approximately 150 nM CDPR. Because the CDPR samples were impure (i.e., we do not know which, if any, of the bands on $NaDodSO_4$–polyacrylamide gels represent CDPR), this value could be considerably lower.

Calcium-dependent proteases are found in many cells and tissues including bovine brain (Drummond & Duncan, 1968). These enzymes have very similar characteristics in all tissues. In addition to the rat liver proteases, we have found that bovine brain CDPR also stimulated calcium-dependent proteases from rat heart, bovine heart, rat erythrocytes, and mouse neuroblastoma cells.[3] Thus, CDPR presumably also stimulates brain calcium-dependent proteases. CDPR was identified in and prepared from bovine brain because of our original interest in calmodulin which is found in high concentrations in brain. We are currently attempting to identify CDPR in other calcium-dependent protease containing tissues such as rat liver. Obviously, if CDPR is an important physiologic regulator of the proteases, it should be located in these same tissues.

Calcium-dependent proteases are cytoplasmic and thus are not segregated from intracellular proteins as are lysosomal proteases. Calcium-dependent proteases may therefore require specialized regulatory mechanisms to control their activities. In addition to calcium, CDPR may provide such regulation. Further work will be required to elucidate the mechanism of this regulation and its possible physiologic significance.

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# Methylation of Ribonucleic Acid in a Cell-Free System from Mouse Myeloma Cells†

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ABSTRACT: Isolated nuclei incorporate few methyl groups into RNA when they are incubated with *S*-adenosyl[*methyl*-$^3H_3$]methionine and four ribotriphosphates. When the nuclei were supplemented with a soluble total cell protein extract, the incorporation of methyl groups into RNA was stimulated 3–6-fold. All classes of RNA were methylated. Methylation of the 2′-OH of ribose and the bases of ribosomal RNA occurred predominantly on endogenous ribosomal RNA precursors, with a minority (20%) occurring on the newly synthesized rRNA precursor. Methylation of the tRNA precursor occurred on both endogenous (40%) and newly synthesized (60%) molecules. The methylation of adenosine in hnRNA occurred predominantly on molecules transcribed in vitro and was sensitive to 1 μg/mL α-amanitin. A final site of methylation was the 7 position of guanosine of the cap structure. About 10% of the RNA polymerase II transcripts were capped in vitro. Capping was blocked 90% by 1 μg/mL α-amanitin and was independent of the presence of the cell protein extract.

Following transcription, most RNAs undergo a series of processing reactions that result in a functional RNA molecule (Perry, 1976). Preribosomal RNAs are methylated on both specific bases and ribose moieties (Salim & Maden, 1973) and specifically cleaved to yield mature ribosomal RNAs. hnRNAs are extensively processed to yield mRNA: the 5′ end of the hnRNA is capped (Shatkin, 1976), the 3′ end of most molecules is polyadenylated (Brawerman, 1974), specific bases are methylated (Perry & Kelley, 1976), and the precursor is specifically cleaved and spliced (Tilghman et al., 1978; Roop et al., 1978; Schibler et al., 1978). The majority of these reactions, particularly on rRNA and mRNA precursors, occur in the cell nucleus, and the maturation of tRNA may also take place, at least partially, in the nucleus (Lönn, 1977; Melton et al., 1980). All classes of RNA are methylated: rRNA predominantly on the 2′-hydroxyl of specific riboses (Maden & Salim, 1974) and hnRNA and mRNA on the "cap" and on the 6-amino group of specific adenosines (Perry & Kelley, 1976; Salditt-Georgieff et al., 1976). Transfer RNA shows the largest variety and greatest number of methyl groups, with a variety of methylated bases as well as *O*-methylribose (Gauss et al., 1979).

Previously we (Marzluff et al., 1973; Cooper & Marzluff, 1978) have described a preparation of cell nuclei from mouse myeloma cells that is active in RNA transcription but that is deficient in some RNA processing reactions. Polyadenylation of RNA (Cooper & Marzluff, 1978) occurs only in the presence of a soluble protein extract derived from crude nuclei. Here we characterize the methylation of RNA. We show enzymes that methylate RNA are easily lost during nuclear preparation. The ability to methylate RNA is restored by the addition of a cell protein extract. A large proportion of the methylation of the tRNA precursor and of hnRNA on internal adenosines occurred on RNA transcribed in vitro. There was capping of the hnRNA in vitro, which was also dependent on transcription. The capping activity was tightly associated with the nuclei.

## Materials and Methods

*Materials.* *S*-Adenosyl[*methyl*-$^3H_3$]methionine and [α-$^{32}$P]GTP were from New England Nuclear. α-Amanitin was from Calbiochem. Actinomycin D was a gift from Merck Sharp & Dohme. Nuclease P1 was from Yamasa Shoyu Co., and other enzymes were obtained from Sigma. Standard methylated nucleosides were obtained from P-L Biochemicals. Triethylamine and phenol were redistilled before use.

*Growth of Cells and Preparation of Nuclei.* Mouse myeloma cells (clone 66-2) were grown in Dulbecco's modified Eagle's media plus 10% horse serum. Cells were harvested at a concentration of (4–6) × $10^5$ mL and nuclei prepared exactly as previously described (Marzluff, 1978).

*Preparation of Cell Extracts.* Cell extracts were prepared by a modification of the method of Cooper & Marzluff (1978). Cells (5 × $10^8$/mL) were homogenized in 0.025 M KCl, 0.002 M $MgCl_2$, 0.01 M Tris,[1] pH 7.5, and 0.001 M DTT. The

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; 6MeAde, 6-methyladenine; 6MeAdo, 6-methyladenosine; DTT, dithiothreitol; hnRNA, heterogeneous nuclear RNA; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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